BUNDESREPUBLIK DEUTSCHLAND

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Prioritätsbescheinigung über die Einreichung einer Patentanmeldung

Aktenzeichen: 199 50 921.2

Anmeldetag: 21. Oktober 1999

Anmelder/Inhaber: BASF Aktiengesellschaft, Ludwigshafen/DE

Bezeichnung: The N-terminal ß-barrel structure of lipid body lipoxygenase mediates its binding to liposomes and lipid bodies

IPC: C 12 N, C 12 P

Die angehefteten Stücke sind eine richtige und genaue Wiedergabe der ursprünglichen Unterlagen dieser Patentanmeldung.

München, den 17. August 2000
Deutsches Patent- und Markenamt
Der Präsident
Im Auftrag

A 916 1
06/00
EDV-L

Patent claims

1. Isolated nucleic acid sequence which codes for a polypeptide and which is composed of a combination of the nucleic acid sequences of a biosynthetic nucleic acid sequence of fatty acid or lipid metabolism and one of the following nucleic acids:

   a) a nucleic acid sequence with the sequence shown in SEQ ID NO: 1,
   b) nucleic acid sequences which are derived as result of the degenerate genetic code of the nucleic acid sequence shown in SEQ ID NO: 1,
   c) derivatives of the nucleic acid sequence shown in SEQ ID NO: 1 which code for polypeptides with the amino acid sequence in SEQ ID NO: 2 and exhibit at least 60% homology at the amino acid level,
   d) a nucleic acid sequence with the sequence shown in SEQ ID NO: 3 or of the amino terminal part of the coding region of this sequence.

2. Isolated nucleic acid sequence according to Claim 1 characterized in that as biosynthesis gene nucleic acid sequence of fatty acid or lipid metabolism a sequence of the following protein groups is used:
acyl-CoA dehydrogenase(s), acyl-ACP (= acyl carrier protein) denature(s), acyl-ACP thioesterase(s), fatty acid acyltransferase(s), fatty acid synthase(s), fatty acid hydroxylase(s), acetyl-coenzyme-A carboxylase(s), acyl-coenzyme-A oxidase(s), fatty acid desaturase(s), fatty acid acetylenase(s), lipoxygenase(s), triacylglycerol-lipase(s), alleneoxid-synthase(s), hydroperoxid-lyase(s) or fatty acid elongase(s).

3. Isolated nucleic acid sequence according to Claim 1 or 2 characterized in that as biosynthesis gene nucleic acid sequence of fatty acid or lipid metabolism a sequence of the following protein groups is used:
fatty acid acyltransferase(s), Δ4-desaturase, Δ5-desaturase, Δ6-desaturase, Δ9-desaturase, Δ12-desaturase, Δ15-desaturase or a fatty acid elongase.

4. Isolated nucleic acid sequence according to Claims 1 to 3 characterized in that at the amino acid level the derivatives named under (c) have an homology of 70%, preferably 80%, most preferably 90% over the whole range of the sequence shown in

SEQ ID NO. 2 (program PileUp, J. Mol.Evolution 25, 351-360, 1987, Higgins et. al., CA-BIOS, 5 1989: 151 - 153).

5. Amino acid sequence coded by a nucleic acid sequence according to Claim 1.

6. Nucleic acid construct containing a nucleic acid sequence according to Claim 1 wherein the nucleic acid sequence is coupled with one or more regulatory signals.

7. Use of a nucleic acid sequence according to Claim 1 or a nucleic acid construct according to Claim 6 for the preparation of transgenic plants.

8. Vector containing a nucleic acid sequence according to Claim 1 or a nucleic acid construct according to Claim 6.

9. Vector according to Claim 8 wherein the vector is a linear DNA or circular DNA, phages, viruses, transposons, IS elements, phasmids, phagemids, cosmids or plasmids.

10. Organisms containing at least one nucleic acid sequence according to Claim 1, at least one nucleic acid construct according to Claim 6, or at least one vector according to Claim 8.

11. Organism according to Claim 10 wherein the organism is a eukaryotic organism.

12. Organism according to Claim 10 or 11 wherein the organism is a plant, a eukaryotic microorganism, or an animal.

13. Organism according to Claims 10 to 12 wherein the organism is a plant, a fungus, or a yeast.

14. Organism according to Claims 10 to 13 wherein the organism is a Yarrowia lypolytica, Saccharomyces, Cereviseae, Traustochytrium, Arabidopsis thaliana, Brassica napus or Linium usitatissimum

15. Transgenic plants containning a nucleic acid sequence according to Claim 1 or a nucleic acid construct according to Claim 6.

16. Method for the targeting of proteins involved in the biosynthesis of lipids or fatty acids in liposomes or lipid bodies characterized in the nucleic acids coding for the

proteins are combined with one of the following sequences in a common protein coding sequence:

a) a nucleic acid sequence with the sequence shown in SEQ ID NO: 1,
b) nucleic acid sequences which are derived as result of the degenerate genetic code of the nucleic acid sequence in SEQ ID NO: 1,
c) derivatives of the nucleic acid sequence shown in SEQ ID NO: 1 which code for polypeptides with amino acid sequence shown in SEQ ID NO: 2 and exhibit at least 60% homology at the amino acid level,
d) a nucleic acid sequence with the sequence shown in SEQ ID NO: 3 or of the amino terminal part of the coding region of this sequence, and

the sequence thus obtained is inserted into a eukaryotic organism.

17. Method for the targeting of proteins involved in the biosynthesis of lipids or fatty acids in liposomes or lipid bodies characterized in that at least one nucleic acid sequence according to Claim 1 or at least one nucleic acid construct according to Claim 6 is inserted into an oil producing organism.

18. Method for the production of fatty acids or lipids characterized in that at least one nucleic acid sequence according to Claim 1 or at least one nucleic acid construct according to Claim 6 is inserted into an oil producing organism, this organism is grown, and the oil contained in the organism is isolated.

19. Method for the production of fatty acids characterized in that at least one nucleic acid sequence according to Claim 1 or at least one nucleic acid construct according to Claim 6 is inserted into an oil producing organism, this organism is grown, and the oil contained in the organism is isolated and the fatty acid is released.

20. Method accodring to Claims 16 to 19 characterized in that the organism is a plant or a eukaryotic microorganism.

The N-terminal β-barrel structure of lipid body lipoxygenase mediates its binding to liposomes and lipid bodies

Christian MAY, Michaela HÖHNE, Petra GNAU, Karsten SCHWENNESEN and Helmut KINDL

Fachbereich Chemie der Philipps-Universität, Hans-Meerwein-Strasse, D-35043 Marburg, Germany

Correspondence to:

Prof. Dr. H. Kindl

Fachbereich Chemie

Philipps-Universität

D-35032 Marburg

Germany

Tel. No. 0049-(0)6421-2822032

Fax No. 0049-(0)6421-2822191

E-mail: hyola@ps1515.chemie.uni-marburg.de

URL: http://www.chemie.uni-marburg.de

Abbreviations. aglutinin, HA; Glutathione S-t[redacted]ferase, GST; Lipid body lipoxygenase, LBLOX; Lipoxygenase, LOX; Phospholipase A_2, PLA.

Enzymes. Glutathione S-transferase (EC 2.5.1.18); isocitrate lyase (EC 4.1.3.1); lipoxygenase (EC 1.13.11.12); phospholipase A_2 (EC 3.1.4.3).

Running title: The β-barrel of lipid body lipoxygenase

Summary

Phospholipase A_2 and a particular isoform of lipoxygenase are synthesized and transferred to lipid bodies during the stage of triacylglyceride mobilization in germinating cucumber seedlings. Lipid body lipoxygenase (LBLOX) is post-translationally transported to lipid bodies without proteolytic modification. Fractionation of homogenates from cucumber cotyledons or homogenates from transgenic tobacco leaves expressing LBLOX showed that a small but significant amount of the LBLOX was detectable in the microsomal fraction. Within the structure of LBLOX, a β-barrel-forming N-terminal domain as deduced from sequence data was shown to be crucial for the selective intracellular transport from the cytosol to lipid bodies. While a specific signal sequence for targetting protein domains to the lipid bodies could not be established it became evident that the β-barrel represents a membrane-binding domain which is functionally comparable to the C2 domains of mammalian phospholipases. The intact β-barrel of LBLOX was demonstrated to be sufficient to target *in vitro* a fusion protein of LBLOX-β-barrel with glutathione S-transferase (GST) to lipid bodies. In addition, binding experiments to liposomes using LOX isoforms, LBLOX deletions and the GST-fusion protein proved the role of the β-barrel as membrane targetting domain. In this respect, the cucumber LBLOX differs from cytosolic isoforms in cucumber and from the soybean LOX-1. If, however, the β-barrel of LBLOX was destructed by insertion of an additional peptide sequence its capacity of targetting proteins to membranes was abolished.

Keywords: β-Barrel, Lip[redacted]body, Lipoxygenase, Phospholipase, [redacted]getting to lipid bodies

Introduction

Oil seed crops play an important role in human nutrition. In germinating seeds, the endogenous reserves of triacylglyceride-storing plants are degraded to allow, even in the absence of light, the de novo formation of new tissue [1,2]. Triacylglycerides are stored in particular highly specialized tissues, *E. g.* in endosperm or cotyledons. Cells of this type contain lipid bodies as the compartments that store the fat [3,4]. At the onset of germination, lipid bodies and their content are degraded providing fatty acids to the glyoxysomes responsible for the fatty acid β-oxidation [5]. In cucumber cotyledons, a phospholipase A_2 (PLA) [6] and a particular isoform of lipoxygenase (lipid body lipoxygenase, LBLOX) [7,8] are synthesized and transferred to lipid bodies during the stage of triacylglyceride mobilization. PLA (a patatin-like protein, [6]) plays a critical role in the initiation of the mobilization process by destructing the phospholipid monolayer of the lipid bodies [9]. Consecutively, the lipoxygenase causes the modification of the acyl moieties in the triacylglycerides which are mostly linoleoyl groups [10]. Eventually, after reduction and the action of a specific hydroxyoctadecadienoyl residues-dependent lipase, S-13-hydroxy-octadecadienoate is released from the lipid bodies to the cytosol [11] and subsequently degraded by glyoxysomes [2,5].

During the course of lipid mobilization, a set of newly synthesized proteins are transferred to the surface of lipid bodies [12], among them LBLOX and PLA. Earlier work [6,7] has shown that LBLOX and PLA are synthesized transiently only during a small period of time, i.e. during the onset of lipid mobilization. The question has to be raised which signals control the intracellular transport of these proteins directed to lipid bodies.

In this paper we provide evidences for a post-translational transfer of LBLOX and PLA to lipid bodies. We demonstrate that an N-terminal domain of lipid body LOX folded as

β-barrel is res ible for the membrane-binding pr ies of the enzyme. In future, this membrane targetting structure may become valuable for oil seeds for its capacity of addressing foreign proteins to lipid bodies.

MATERIALS AND METHODS

Plant material and cell fractionation

Cucumber seeds were germinated in the dark at 26 °C for 2 or 4 days as indicated for each particular preparation. Cotyledons were harvested and homogenized by cutting with a scalpel as described earlier [13]. Following removal of cell debris and differential centrifugation, the pellets either of the 10 000 **g** centrifugation or the 100 000 **g** centrifugation were subjected to sedimentation or flotation in a sucrose gradient according to [14]. For a crude lipid body fraction, the supernatant of a short (10 min) centrifugation at 2 000 **g** was subjected to centrifugation at 10 000 **g** for 30 min, and the lipid layer formed on the top of the centrifuge tube was collected. Further purification of lipid bodies was by gentle suspending the lipid layer and repeated re-flotation [12].

Tobacco plants were cultivated in Magenta boxes at 22 °C under continuous light (2 000 lux). The media for growing transformants were according to [15,16].

Plasmid constructions and preparations of proteins

For preparing deletion forms of LBLOX, we used CSLBLOX-221 contained in vector pSport-1 [8]. An N-terminal deletion was constructed by cleaving CSLBLOX-221 first with *SmaI*/*NdeI* followed by a cut with *HaeIII*. Following ligation into pSport-1 (Life Technologies), this construct deleted in the first 80 nucleotides of pCSLBLOX-221 [8] was designated LBLOXΔ80N. After preparation of mRNA by transcription *in vitro*, the translation of the respective protein started at a methionine residue corresponding to nucleotide 192 of pCSLBLOX-221 and the protein thus lacked the 48 N-terminal amino acid residues of wild type LBLOX. LBLOXΔ51 and LBLOXΔ96 were both deletions in the C-

terminal region downstream of the amino acid residue 696. LBLOXΔ51 and LBLOXΔ96 contained deletions of 51 or 96 amino acid residues following the position 696, but possessed the original C-terminus. For those preparations, the *Msc*I site at nucleotide 2128 of pCSLBLOX-221 was used for cleavage and the upstream fragment was ligated to the respective C-terminal fragments created by PCR. For the construction of LBLOXΔ504, the *Nde*I site of pCSLBLOX-221 at nucleotide 1450 and the *Aat*II site downstream of the translation stop of pCSLBLOX-221 were used for deleting the whole C-terminal part of LBLOX. After blunt-ending and religation of these sites, the respective protein prepared by *in vitro* transcription/translation of the construct lacked the C-terminal half of the LBLOX molecule.

Cloning of a fusion protein, GST-LBLOX244, was performed by using as N-terminal part a fragment originating from the 26 kDa glutathione S-transferase domain [17] from *Schistosoma japonicum,* and as C-terminal part the 244 amino acid residues-containing N-terminal portion of LBLOX. Using the *Bam*HI/*Xho*I-cleaved gene fusion vector pGEX-4T-3 (Pharmacia) and a 732 nucleotide fragment corresponding to amino acid residues 1-244 of LBLOX for cloning and the affinity tag for protein purification, the fusion protein GST-LBLOX244 was isolated by chromatography on Glutathione Sepharose 4B (Pharmacia).

Bacterial expression of LBLOX was carried out after cloning the insert of pCSLBLOX-221 into a pQE-30 vector (Qiagen). For preparing LBLOX by *in vitro* translation, the LBLOX sequence contained in vector pSport-1, pCSLBLOX-221 [8], was cleaved with *Aat*II, transcribed using T7 polymerase and translated in a reticulocyte lysate. Similarly, the patatin-like protein (identical to phospholipase A_2) was obtained by *in vitro* transcription/translation using pCSPAT-291 and the PLA cDNA under the control of the T7 promotor [6].

Transfection tobacco

Using the vector pBI121 (Clontech) containing the 35S-RNA promoter of caulimo virus, the coding region of the β-glucuronidase gene and the NOS terminator we prepared the construct pBI121ΔGUS by cleaving out the GUS cassette with *Sma*I and *Sst*I and forming a blunt end using T4 DNA polymerase. LBLOX contained in vector pSport-1 [8] was cleaved with *Sma*I/*Bam*HI, ligated with a *Bam*HI linker and introduced into the *Bam*HI-cut dephosphorylated vector pBI121ΔGUS. The product, pBI121ΔGUS-LBLOX, was either used for transformation into *Agrobacterium tumefaciens* or further modified to pBI121ΔGUS-LBLOX-HA_3. The construction of the later plasmid was carried out by using a unique *Sac*I site following nucleotide 252 of pCSLBLOX-221. Following the cleavage with *Sac*I and dephosphorylation, a three-fold hemaglutinin-tag (HA-tag) furnished with a *Sac*I site was inserted. Taking into account 3 stretches of -YPYDVPDYA- and linkers, the total length of the insert amounted to 30 amino acid residues.

Agrobacterium tumefaciens LB-A4404 was transformed with pBI121ΔGUS-LBLOX or pBI121ΔGUS-LBLOX-HA_3 by the freeze-thaw method. These bacteria were used to transform leaf discs of *Nicotiana tabacum* cv. Petit Havanna SR-1 according to the established procedure of Horsch *et al.* [15]. Shoots were selected on Linsmaier and Skoog medium supplemented with 0.5 mg/L N-benzylaminopurine, 500 mg/L cefotaxim, and 75 mg/L kanamycin. Kanamycin-resistant plants were tested for the presence of the LOX DNA by PCR and Northern blot analysis. Positive plants with elevated LOX levels were propagated vegetatively.

Preparation of HA-tagged LBLOX by expression in tobacco

Leaves of homozygous kanamycin-resistant tobacco lines containing the LBLOX-HA_3 construct were homogenized in 50 mM Hepes-NaOH, pH 7.4, 5 mM $MgCl_2$, 0.5 mM EDTA, 50 mM KCl, 2.5 mM dithiothreitol, 2 mM phenylmethylsulfonylfluoride, and 15% (w/w) sucrose (buffer A) in the presence of polyvinylpyrrolidone (25 000) (Merck).

Following centrifugation the supernatant of the 100 000 g centrifugation was desalted, concentrated and fractionated on a large Biogel A-1.5 column. Fractions representing the 100 kDa region and analyzed by Western blots using anti-HA antiserum were collected. This mutant LBLOX protein contained, following the amino acid residue 68 of the wild-type enzyme, an insertion of 30 amino acid residues equivalent to a three-fold HA-tag and had a theoretical molecular mass of 104 kDa. This difference in size between wild type enzyme and recombinant protein was clearly visualized on SDS-PAGE.

Radioactive labelling of proteins by synthesis *in vitro*

Translation was carried out using purified mRNAs, reticulocyte lysate and [^{35}S]L-methionine in the presence or absence of dog pancreas microsomes. Alternatively, microsomes prepared from cucumber cotyledons [14] were used for co-translational or post-translational transport assays.

Labelling of proteins by protein synthesis *in vivo*

Radiolabelling experiments *in vivo* were performed as short pulse experiments with cotyledons of seedlings germinated for 4 d. Five g of cotyledons were cut into 2 mm slices and incubated with 8 MBq [^{35}S]L-methionine (40 TBq/mmol) for 15 min. Cautious homogenization and preparation of subfractions was carried out as described above.

Administration of $^{45}Ca^{2+}$ and analysis of the lipid body fraction

Two g of cotyledons collected from cucumber seedlings grown for 2.5 d in the dark at 26 °C were cut into slices and incubated in the dark with 6 MBq $^{45}Ca^{2+}$ (8 GBq/mmol) for 3 h. Following homogenization by chopping with a scalpel in the presence of buffer A, the homogenate was centrifuged for 25 min at 2 000 g. After removal of the top layer, containing lipid bodies, and decanting from the sediment, the extract was subjected to centrifugation at 100 000 g for 1 h.

The fraction containing lipid bodies was resuspended in buffer A, adjusted to 30% (w/w) sucrose, and overlayed with buffer A. Following centrifugation at 100 000 **g** for 1 h, the lipid bodies floating up were collected and subjected to various washing procedures.

Preparation of liposomes

Liposomes were prepared as large unilamellar vesicles according to [18] either from a crude soybean lecithine mixture (Sigma) or from defined dilinoleoyl-phosphatidylcholine, with or without the addition of the serine derivative. In routine work applying detergent solubilization and removal, a molar ratio of dilinoleoyl-phosphatidylcholine to sodium cholate was 0.6 : 1.0. The efficiency of detergent removal by dialysis in the Amicon chamber equipped with PM10 membrane was controlled [19]. The size of the vesicles was checked under the microsope by comparison with the size (10 ±0.5 µm) of MonoQ beads (Pharmacia). For particular experiments, triacylglycerides (trilinolein) were included in the liposomes to obtain phospholipid-covered lipid droplets comparable to "black" lipid bodies. This preparation was started by dissolving 200 mg soybean lecithin and 500 mg trilinolein in 10 mL methanol/chloroform (1:1). After removing the solvent *in vacuo*, the residue was suspended in dialysis buffer according to [19]. In any case, the vesicles recovered by floatation were analyzed by TLC. The diameter of the liposomes was in the range of 1 µm.

Binding experiments in combination with flotation assay

A suspension of liposomes corresponding to 1 mg lecithin in 150 mM NaCl, 50 mM Tris-HCl, pH 8,0, was incubated for 10 min either with 4 µg unlabelled protein or with the supernatant of a reticulocyte lysate translation mixture. After adjusting to 42% (w/w) sucrose, the suspension was placed into a 12-mL centrifuge tube. A linear sucrose density gradient ranging from 37-26% (w/w) sucrose was placed on top of the sample. Flotation

of the protein-covered [redacted]omes was by centrifugation at 100 g for 6 h. Following fractionation, the protein analysis was by SDS-PAGE and immunodecoration.

Binding experiments with lipid bodies or microsomes were performed in an analogous way. All densities within the sucrose gradients are given in correlation to sucrose concentration (w/w).

Immunological techniques

Antisera were raised in rabbits against LBLOX [12], PLA [6], and isocitrate lyase [20]. In addition, monoclonal antibodies against GST (Pharmacia) and the epitope from human influenza hemaglutinin protein (Boehringer) were used. Immunoprecipitations of radioactively labelled enzymes were performed under standardized conditions (procedure 1) by adding 1 µg of the respective purified protein to the mixture prior to precipitation using 20 µL antiserum. Following standing for 12 h at 20 °C and 20 h at 4 °C the precipitate was sedimented by centrifugation at 3 000 g. The pellet was washed at least 5-times and then dissolved in SDS. For indirect precipitation of other proteins (procedure 2), the antigen was not further diluted but incubated with 2 µL antiserum for 6 h, and subsequently mixed with protein-A-Sepharose. After transfer of the mixture into a small tube and extensive washing, the antigen was eluted, together with the IgGs, using 100 mM acetic acid.

Other assays

For comparing protein structures, limited proteolysis was carried out in the gel [8]. TLC analysis of lipid was carried out on silica gel G (Merck) using methanol-chloroform-water, 65:25:4, as solvent system.

RESULTS

To overview the sequence of steps required for the transfer of LBLOX from the ribosome to the final cellular destination we first investigated which pools within the cell are passed by the LBLOX protein. Second, we tried to localize the targetting structure, sequence or domain, responsible for the transfer.

Investigations with LBLOX *in vivo* and *in vitro* to distinguish between co-translational transport to the ER and post-translational transport to lipid bodies

No differences were observed between the molecular mass of *in vitro* translated LBLOX and the membrane bound cellular forms, on the ER or lipid bodies (Fig. 1A). This indicates that the transport to the membranes takes place without apparent chemical modifications. Both a co-translational transport to the ER followed by a subsequent transfer to lipid bodies, and a post-translational transport to lipid bodies via a cytosolic pool were likely mechanisms how LBLOX reaches its final intracellular site. First, translation of LBLOX mRNA *in vitro* was performed under conditions of co-translational transport in the presence of microsomes from dog pancreas or microsomes from cucumber cotyledons. Second, a similar protocol was applied to test whether radioactively labelled LBLOX translatate was also transferred to the membranes if translation and addition of microsomes were performed consecutively (Fig.1B). In the later case, ribosomes were removed by centrifugation from the *de novo* synthesized protein prior to the addition of microsomes. Following re-isolation of ER-enriched membranes by flotation, only a portion of the translated protein remained at the position where the suspension was placed within the gradient (Fr. No. 12). Some LBLOX migrated to densities corresponding to 33% sucrose (Fr. No. 11) or to 30% sucrose (Fr. No. 9). Small amounts of LBLOX were recovered at the position of the smooth ER (Fr. Nos. 5-7).

By comparing in a series of experiments the amount of LBLOX radioactivity in the membranes re-isolated by gradient centrifugation it became evident that using the set-up

for post-translational sport to the microsomes yielded the same or slightly higher amounts of membrane-bound LBLOX than using the cotranslational transport protocol (data not shown). Precisely comparing the migration behaviour of the primary LBLOX translatate and the LBLOX re-isolated from the membranes after flotation we did not find discernible differences in molecular mass.

Similarly, we translated PLA mRNA obtained by *in vitro* transcription of pPAT-291 and incubated the translation product, i.e. radioactively labelled phospholipase, with microsomes. Following flotation in a sucrose gradient, the subfractions were analyzed by SDS-PAGE and fluorography (Fig. 1C). The fluorography revealed that a large portion of the translation product bound to microsomal membranes.

These results obtained by studying the *in vitro* LBLOX transfer to microsomes (Fig. 1B) were corroborated by analyzing the situation *in vivo* by following the sequences of events taking place in the transfer of LBLOX to lipid bodies. Applying pulse-chase experiments we set out to define the pools which were passed by the newly synthesized LBLOX on its way to lipid bodies. Thus, we wanted to know whether either the fraction of the ER membranes or the cytosol represented the pool where labelled LBLOX first appeared.

Fig. 2A (lane 2) shows that the main portion of pulse-labelled LBLOX was recovered in the fraction containing cytosol. However, a low but significant amount of radioactive LBLOX was consistently found in microsomes and also in microsomal fractions further purified by flotation in sucrose gradients (Fig. 2A). To exclude the possibility that a small percentage of all *de novo* synthesized labelled proteins contaminated the ER-containing fraction we analyzed in the microsomal fraction the amount of proteins which were either cytosolic or are artefactually released into the cytosol-containing supernatant of a 100 000 g centrifugation. Using isocitrate lyase as highly expressed cucumber protein at this developmental stage, the presence of this protein in the microsomal fraction and in the soluble supernatant was determined by

immunoprecipit . Fig. 2B shows that isocitrate [redacted]se, other than LBLOX, was virtually absent from the ER preparation.

The data presented in Fig. 2A indicate that LBLOX passes the cytosol as first pool on its way to the lipid bodies. It became also evident that LBLOX possesses membrane-binding properties and is partially protected from proteolysis when bound to the ER membrane. Despite of stretches of the LBLOX molecule showing affinity to membranes a large portion of LBLOX *in vitro* is accessible to chemical modification and may therefore *in vivo* extend into the cytosol. Part C of Fig. 2 provides some indication to what extend the portion of LBLOX that is bound to microsomal membranes is accessible to proteolytic degradation.

The LOX isoform detectable by Western blot analysis on microsomes isolated from cotyledons was found to be structurally identical to the LOX isoform bound to lipid bodies. This was evidenced by comparing the fragment pattern after limited proteolysis (data not shown). The kind of binding between the microsomal membrane LBLOX is the one of a peripheral bound membrane protein. Washings with 100 mM $MgCl_2$ removed more than 90% of the labelled LBLOX. However, it should be emphasized that the binding of LBLOX to the microsomes in the presence of low salt buffer was rather stabile, also under the condition of repeated gradient centrifugation. The stabile association was also demonstrated by showing that the LOX on microsomes was only partially accessible for proteolysis (Fig. 2, part C).

The capacity of binding to microsomal membranes was demonstrated for LBLOX also under another *in vivo* condition. In a heterologous system, *i. e.* green leaves of tobacco expressing LBLOX of cucumber cotyledons, the high expression of LBLOX led to the binding of substantial amounts of LBLOX to microsomes. By subfractionation of the 100 000 **g** pellet applying a sucrose density gradient flotation we demonstrated that virtually all LBLOX previously sedimented by centrifugation at 100 000 **g** remained bound to membranes floting up. The peak of membrane-bound LBLOX (Fig. 2, part D) coincided with marker proteins for ER membranes.

To extend our list of evidences for a direct post-translational transfer of LBLOX to membranes of lipid bodies we studied *in vitro* the membrane affinity of soluble LBLOX to microsomes. In a mixing experiment, we added a mixture of radioactive cytosolic proteins prepared from cotyledons after pulse labelling (prep A) to a supernatant of an extract prepared from unlabelled cotyledons and subjected to a 2 000 **g** centrifugation (prep B). This procedure should allow the radioactive LBLOX contained as precursor in the cytosolic and membrane-free preparation (prep A) to bind post-translationally to microsomes, lipid bodies and other membranes present in the non-radioactive extract (prep B). After a short incubation, we isolated microsomes as potential acceptor membranes for radioactive LBLOX (Fig. 3A), and glyoxysomes as control (Fig. 3B). The electrophoretic analysis showed that cytosolic LBLOX became bound to microsomes also under these conditions resembling the *in vivo* situation. In contrast, LBLOX was not detectable in the glyoxysomal fraction subjected to a final purification by sucrose gradient flotation. Thus, soluble LBLOX binds to membranes but not to all membranes equally. *E. g.*, the affinity of LBLOX to glyoxysomal membranes (Fib. 3B) is at least more than one order of magnitude lower than the affinity demonstrated to microsomes (Fig. 3A, lane 3). Therefore, binding of LBLOX in cucumber cotyledons requires a high degree of selectivity because other organelles are not decorated with LBLOX, and are not modified for degradation.

Cucumber LBLOX and soybean LOX-1 differ in their affinity to liposomes

To test whether LBLOX possesses an intrinsic affinity to membrane lipids, independent of specific protein-protein interactions, we extended our binding assays by including liposomes as acceptor membranes. First, the experiments were performed with crude soybean lecithin as source of phosphatidylcholine. Subsequently, phosphatidylcholine of various degrees of purity, also in combination with phosphatidylserine, were used for the

preparation of lip[redacted]nes. The size of liposomes was con[redacted]d by comparison with MonoQ beads as standard.

The results of the liposome experiment (Fig. 4) reveal the marked difference between the membrane affinity of cucumber LBLOX and the one of soybean LOX-1. While the two proteins previously attributed to cucumber lipid bodies, namely LBLOX and PLA, were almost quantitatively bound to the liposomes the cytosolic LOX forms of cucumber and soybean LOX-1 lacked an affinity to the lipid phase. In control experiments either with a typical cytosolic protein or with a mixture containing LBLOX and cytosolic proteins prepared from cucumber cotyledons we could demonstrate that the membrane affinity of LBLOX is rather unique (data not shown).

Evidence that the N-terminal region including the β-barrel structure is essential for binding LBLOX to lipid bodies

Considering the lack of proteolytic processing and taking into account the data obtained with binding studies the hypothesis may arise that domains of a folded protein are responsible for the transfer to the membranes. Such a domain became likely when the amino acid sequence of LBLOX was fitted into a structure based on the crystal structure data obtained for soybean LOX-1 at a 1.4 Å resolution [21]. Other than soybean LOX-1, the cucumber LBLOX showed not only a β-barrel structure shortly downstream of the N-terminal part but had also within this domain a rather unique arrangement of glutamyl residues which could be used for co-ordination of Ca^{2+} (Fig. 5). This in turn may point to a relationship between the LBLOX β-barrel and the members of Ca^{2+}-dependent membrane binding domains.

To test the concept of a single domain as means of protein targetting we constructed a cDNA coding for a fusion protein consisting of glutathione S-transferase and the N-terminal β-barrel derived from LBLOX as C-terminal part. Following expression in bacteria, a 51 kDa protein was isolated containing the 220 amino acid residues which

originate from the glut[redacted]ne S-transferase (representing th[redacted]utathione binding site) and the 244 N-terminal amino acid residues of LBLOX.

Employing 1 μg of the GST-LBLOX244 fusion protein and a lipid body suspension corresponding to 20 μg protein we observed an almost quantitative transfer of the fusion protein to lipid bodies, on top of the LBLOX already present on the lipid bodies. The high extent with which the β-barrel fusion protein was attached to the lipid body surface indicates that either the fusion protein competed well with the LBLOX originally bound to the lipid bodies or the lipid body surface was not fully saturated with LBLOX. Fig. 6 summarizes the evidence that the N-terminal β-barrel of LBLOX alone is sufficient for a very efficient binding to lipid bodies. In lane 5 the uptake of the 51 kDa fusion protein by the lipid bodies is demonstrated.

Two cDNA constructs, LBLOXΔ51 and LBLOXΔ96, were prepared, and after *in vitro* transcription/translation the respective proteins were tested whether they bind to isolated lipid bodies. Both recombinant proteins were efficiently transferred to lipid bodies (data not shown). These experiments were designed because the analysis of the hydropathy plots of the LBLOX amino acid sequence indicated that a stretch of amino acid residues around position 710 carries a hydrophobic region which might be sufficient for a potential membrane-association. However, the binding assays were positive implying that the lack of the 710 region, created in the constructs LBLOXΔ51 and LBLOXΔ96, does not significantly decrease the efficiency of LBLOX binding to lipid bodies. Using *in vitro* assays as shown in Fig. 1B, we also found that radioactively labelled translatate of LBLOXΔ504 was transported to isolated lipid bodies. LBLOXΔ504 consisted only of the N-terminal half of the LBLOX molecule. This further corroborates the concept that the C-terminal part of LBLOX which encompasses the active site is not necessary for the targetting. In contrast, LBLOXΔ80N lacking the N-terminal extension but possessing the β-barrel was transferred to lipid bodies with significantly less efficiency than LBLOXΔ504.

By exten this kind of *in vitro* experiments we nd that the transfers to lipid bodies take place in a Ca^{2+}-independent way (data not shown). Despite this result, we investigated the possibility that Ca^{2+}-dependent binding of proteins to the lipid body surface may eventually cause the formation of a Ca^{2+}-enriched protein coat surrounding the lipid bodies. To study the recruitment of Ca^{2+} together with proteins like LBLOX and PLA, we examined the formation of Ca^{2+}-covered lipid bodies by administering $^{45}CaCl_2$ to cotyledons and subsequent isolation of cell structures. The lipid body fraction contained, following reflotation, washing and treatment with 100 mM Na_2CO_3, 50 kBq $^{45}Ca^{2+}$ corresponding to appr. 1 nmol. The same preparation possessed LBLOX amounting to 2 nmol. Upon further treatment with 100 mM unlabelled $CaCl_2$, 90% of the radioactivity was removed from the lipid bodies while most of the LBLOX remained bound to the lipid bodies. These data are not in agreement with the hypothesis that Ca^{2+} is a prerequisite of LBLOX binding to the lipid body surface.

Substantial alteration in the β-barrel of LBLOX abolishes its capacity of binding to liposomes

To further characterize the kind of interaction between the lipid body surface and the N-terminal β-barrel structure of LBLOX we investigated whether a modified β-barrel structure also binds to liposomes. To this end, a recombinant protein (LBLOX-HA_3) was prepared containing, compared to the wildtype LBLOX, a threefold hemeagglutinin-tag inserted between amino acid residue 70 and 71 of LBLOX. This construction interrupts the β-barrel by a stretch of 30 amino acid residues.

The liposome experiments shown in Figure 7 summarize the evidence that the N-terminal β-barrel of LOX alone is sufficient for its binding to membranes and that the destruction of the β-barrel inactives this transfer. While wild-type LBLOX and the β-barrel fusion protein (GST-LOX) virtually quantitatively bind to the liposomes, the insertion of a peptide into the barrel structure abolishes the membrane affinity.

DISCUSSION

A few isoforms of lipoxygenase bind or integrate into membranes of various organelles. In such cases, the function of the lipoxygenase may be directed towards the modification of the respective membrane. The expression of 15-LOX in reticulocytes reaches maximal values immediately before organelle degradation [22] as does lipid body LOX in fat-degrading cotyledons [7,23]. Thus, in several types of eukaryotic cells the function for LOX lies in a programmed organelle degradation [24]. In the case of LBLOX, both the phospholipid monolayer and the bulk of triacylglycerides are modified resulting in the formation of 13-S-hydroperoxyoctadecadienoyl moieties [10,11] which in turn initiates the mobilisation of the storage lipid. For this action of LBLOX, its binding to the target membrane might be a prerequisite.

Binding experiments *in vitro* and cell fractionation studies describing the situation *in vivo* were performed to characterize the steps required for the directional intracellular transport of LBLOX to lipid bodies. The results of both kinds of experimental set-up support the concept that a cytosolic pool of the primary translation product exists and that the consecutive transfer to lipid bodies occurs post-translationally. As the target organelles are already present at the stage of seed germination, the attachment of the lipid-degrading enzymes, namely LBLOX and PLA, to the organelle thus depends primarily on the transient expression of the genes coding for the particular isoforms of LOX and PLA. The earlier findings of a temporal pattern of LOX [7] and PLA [6] expression in the cotyledons are consistent with their stage-dependent role in the degradation of lipid body structures.

Noteworthy, the binding observed with LBLOX is not a transient association but a stabile binding at microsomes and lipid bodies. The strength of this binding was evidenced by the recovery of LBLOX with lipid bodies or liposomes following the stringent separation by flotation of excess ligand and acceptor membranes. However, this binding is not comparable with the integration of a protein containing a long stretch of hydrophobic

amino acid residues as demonstrated for oleosins, another form of lipid body proteins [25], it is rather similar to the behaviour of peripheral membrane proteins. As for the later case, it might be worth discussing whether the LBLOX binding is due to its interaction with an integral membrane protein as partner, *E. g.* with the oleosin as anchor, or whether the function of the LBLOX β-barrel is comparable to the function of a phospholipid membrane binding C2 domain as shown in synaptotagmin [26] and cytosolic phospholipase C-delta [27] or phospholipase A_2 [28]. This kind of comparison with C2 domains requires a thorough investigation of a potential Ca^{2+}-effect either on the membrane-binding properties of LBLOX or on the structure of LBLOX as found for 5 - lipoxygenase [29]. During the course of our experiments with lipid bodies, $^{45}Ca^{2+}$-coating of lipid bodies was found but a Ca^{2+}-dependence for the binding of LBLOX constructs was not observed. However, for an unequivocal prove or disprove of a Ca^{2+}-mediated binding, for future studies the experimental protocols have to be refined in several aspects. Previous experiments [30] with root tissue and using energy-filtering electron microscopy have provided evidence that the lipid body surface is covered by a zone of high Ca^{2+} content.

We should recall that the comparison of primary amino acid sequences and the putative secondary structure of LOX forms shows that LBLOX is distinct not only by the formation of a β-barrel structure furnished with exposed glutamyl residues but also by an N-terminal extension of 30 amino acid residues which is not present in cytosolic isoforms of LOX [8,31]. Thus, it is likely that both kinds of interactions play a crucial role, the N-terminal extension as specific motif and the β-barrel, positioned in the amino acid sequence subsequent to the N-terminal extension, as general means of increasing membrane affinity.

Our experiment with the LBLOX mutant protein differing from the wild type only by possessing a substantial alteration in the β-barrel, *i. e.* by the insertion of three repeats of a HA-tag, demonstrated the loss of association to lipid bodies or membranes.

This means that the in β-barrel must be considered as es[redacted]tial for the binding to lipid bodies irrespective whether an additional selectivity is contributed by the far N-terminal region.

Targetting signals as unfolded stretches of amino acid residues have been recognized in cases where proteins are transferred to mitochondria, chloroplasts or the ER. Here, in the case of protein association to pre-existing lipid bodies, a domain already folded in a distinct form may cause the membrane binding. An additional part has to be postulated which confers selectivity to the binding of LBLOX to lipid bodies. If a high level of LBLOX is present, the ER and Golgi vesicles are covered with LBLOX, in addition to lipid bodies. If, however, the intracellular level of LBLOX decreases the LBLOX molecules will occupy mainly the surface of lipid bodies.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 286).

REFERENCES

1. Ap Rees, T., Thomas, S. M., Fuller, W. A. & Chapman, B. (1975) Location of gluconeogenesis from phosphoenolpyruvate in cotyledons of *Cucurbita pepo*. *Biochim. Biophys. Acta* **385**, 145-156.
2. Kindl, H. (1993) Fatty acid degradation in plant peroxisomes: Function and biosynthesis of the enzymes involved. *Biochimie* **75**, 225-230.
3. Murphy, D. J. (1993) Structure, function and biogenesis of storage lipid bodies and oleosins in plants. *Prog. Lipid Res.* **32**, 247-280.
4. Huang, A. H. C. (1994) Structure of plant seed oil bodies. *Curr. Opinion Struct. Biol.* **4**, 493-498.
5. Kindl, H. (1997) The oxygen-dependent modification of triacylglycerols and phospholipids, the different way of initiating lipid body mobilization. *Z. Naturforsch. C* **52**, 1-8.
6. May, C., Preisig-Müller, R., Hohne, M., Gnau, P. & Kindl, H. (1998) A phospholipase A_2 is transiently synthesized during seed germination and localized to lipid bodies. *Biochim. Biophys. Acta* **1393**, 267-276.
7. Feussner, I., Nellen, A., Hause, B., Wasternack, C. & Kindl, H. (1996) Lipid body lipoxygenase is expressed in cotyledons during germination prior to other lipoxygenase forms. *Planta* **198**, 288-293.
8. Höhne, M., Nellen, A., Schwennesen, K. & Kindl, H. (1996) Lipid body lipoxygenase characterized by protein fragmentation, cDNA sequence and very early expression of the enzyme during germination of cucumber seeds. *Eur. J. Biochem.* **241**, 6-11.
9. Noll, F., May, C. & Kindl, H. (1999) Phospholipid monolayer of plant lipid bodies attacked by phospholipase A_2 shows 80 nm holes analyzed by atomic force microscopy. *J. Struct. Biol., submitted.*

10. Feussner, I., Ba[redacted]enn, A., Höhne, M. & Kindl, H. (199[redacted]l three acyl moieties of trilinolein are efficiently oxygenated by recombinant His-tagged lipid body lipoxygenase *in vitro*. *FEBS Lett.* **431**, 433-436.

11. Feussner, I., Wasternack, C., Kindl, H. & Kühn, H. (1995) Lipoxygenase-catalyzed oxygenation of storage lipids is implicated in lipid mobilization during germination. *Proc. Natl. Acad. Sci. USA* **92**, 11849-11853.

12. Sturm, A., Schwennesen, K. & Kindl, H. (1985) Isolation of proteins assembled in lipid body membranes during fat mobilization in cucumber cotyledons. *Eur. J. Biochem.* **150**, 461-468.

13. Kindl, H. & Kruse, C. (1983) Biosynthesis of glyoxysomal proteins. *Methods Enzymol.* **96**, 700-715.

14. Sturm, A. & Kindl, H. (1983) Fucosyl transferase activity and fucose incorporation *in vivo* as markers for subfractionating cucumber microsomes. *FEBS Lett.* **160**, 165-168.

15. Horsch, R. B., Fry, J. E., Hoffmann, N. L., Eichholtz, D., Rogers, S. G. & Fraley, R. T. (1985) A simple and general method for transferring genes into plants. *Science* **227**, 1229-1231.

16. Jefferson, R. A., Kavanagh, T. A. & Bevan, M. W. (1987) GUS-fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901-3907.

17. Smith, D. B. & Johnson, K. S. (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* **67**, 31-40.

18. Woodle, M. C. & Papahadjopoulos, D. (1989) Liposome preparation and size characterization. *Methods Enzymol.* **171**, 193-217.

19. Yamazaki Kodama, M. & Gabius, H.J. (1994) glycoprotein-liposome and lectin-liposome conjugates as tools for carbohydrate recognition research. *Methods Enzymol.* **242**, 56-65.

20. Frevert, J. & Kindl, H. (1978) Plant microbody proteins. Purification and glycoprotein nature of glyoxysomal isocitrate lyase from cucumber cotyledons. *Eur. J. Biochem.* **92**, 35-43.

21. Minor, W., Steczko, J., Stec, B., Otwinowski, Z., Boldin, J. T., Walter, R. & Axelrod, B. (1996) Crystal structure of soybean lipoxygenase L-1 at 1.4-A resolution. *Biochem.* **35**, 10587-10701.

22. Kühn, H., Balkner, J., Wiesner, R. & Brash, A. R. (1990) Oxygenation of biological membranes by the pure reticulocyte lipoxygenase. *J. Biol. Chem.* **265**, 18351-18361.

23. Matsui, K., Hijiya, K., Tabuchi, Y. & Kajiwara, T. (1999) Cucumber cotyledon lipoxygenase during postgerminative growth. Its expression and action on lipid bodies. *Plant Physiol.* **119**, 1279-1287.

24. van Leyen, K., Duvoisin, R. M., Engelhardt, H. & Wiedmann, M. (1998) A function for lipoxygenase in programmed organelle degradation. *Nature* **395**, 392-395.

25. Hills, M. J., Watson, M. D. & Murphy, D. J. (1993) Targetting of oleosins to the oil bodies of oilseed rape (*Brassica napus* l). *Planta* **189**, 24-29.

26. Rizo, J. & Sudhof, T. C. (1998) C-2-domains, structure and function of a universal Ca^{2+}-binding domain. *J. Biol. Chem.* **273**, 15879-15882.

27. Perisic, O., Fong, S., Lynch, D. E., Bycroft, M. & Williams, R. L. (1998) Crystal structure of a calcium-phospholipid binding domain from cytosolic phospholipase A_2. *J. Biol. Chem.* **273**, 1596-1604.

28. Xu, G. Y., McDonagh, T., Yu, H. A., Nalefski, E. A., Clark, J. D. & Cumming, D. A. (1998) Solution structure and membrane interactions of the C2 domain of cytosolic phospholipase A_2. *J. Mol. Biol.* **280**, 485-500.

29. Hammarberg, T. & dmark, O. (1999) 5-Lipoxygenase b calcium. *Biochem.* **38**, 4441-4447.

30. Busch, M. B., Kortje, K. H., Rahmann, H. & Sievers, A. (1993) Characteristic and differential calcium signals from cell structures of the root cap detected by Energy-Filtering electron microscopy (EELS/ESI). *Eur. J. Cell Biol.* **60**, 88-100.

31. Rosahl, S. (1996) Lipoxygenases in plants - their role in development and stress response. *Z. Naturforsch.* **C 51**, 123-138.

Legends to Figures

Fig. 1. Comparison of LBLOX forms prepared *in vitro* by translation and LBLOX isolated from cell fractions. Part A (fluorography) shows a comparison of the migration behaviour of LBLOX prepared by *in vitro* translation (lane 1), LBLOX isolated from microsomes (lane 2) and LBLOX isolated from lipid bodies (lane 3). For lane 2 and 3, the respective cellular subfractions were prepared from cotyledons following protein labelling *in vivo* using [^{35}S]L-methionine. Part B and C present fluorographies showing the post-translational binding to isolated cucumber microsomes. Cucumber microsomes were incubated with the radioactively labelled proteins formed in reticulocyte lysates governed either by LBLOX mRNA or PLA mRNA and subsequently purified by flotation in a density gradient. The membrane-bound lipid body lipoxygenase (part B) or phospholipase (part C) recovered after flotation are shown on the left side. Lane 1 in B and C corresponds to the top of the sucrose gradient while lane 12 (in B) and lane 13 (in C) correspond to the bottom and represent the proteins not bound to membranes (not floting) remaining at the position where the incubation mixture was placed underneath of the gradient prior to centrifugation.

Fig. 2. Results of pulse labelling of proteins in cotyledons indicating a weak binding of LBLOX to microsomes but a substantial pool of LBLOX in the cytosol. After a short period of time (15 min) used for the administration to cotyledons of the radioactive amino acid as precursor, a cell fractionation was performed and two proteins were isolated from the cellular subfractions by immunoprecipitation. The isolation of the radioactive proteins from the solubilized subfractions was carried out after adding as carrier 1 µg of the respective cold protein (LBLOX or isocitrate lyase, ICL) by addition of the antiserum and direct precipitation (see Methods). After electrophoresis and protein stain (lane 3: microsomes; lane 4: cytosol), the fluorography (lanes 1 and 2) in part A indicated that LOX was extensively labelled in the cytosol (lane 2) and much less in the microsomes (lane 1). Part B: As control, the distribution of

isocitrate lyase betwee[redacted]se two fractions was determined. 1 (microsomes) and lane 2 ("cytosol") show fluorographies, while lanes 3 and 4 represent the protein stains. Lane 1 (and the corresponding protein stain in lane 3) shows the absence of contamination in microsomes by isocitrate lyase. Part C: Treatment of labelled microsomes (as analyzed in part A, lane 1) with proteinase K. Subsequent to the proteolysis, phenylmethylsulfonylfluoride and 1 µg of the respective cold protein were added, and the immunoprecipitation performed. Lanes 1 to 4 represent fluorographies: microsomes untreated, in lane 1; cytosol untreated, in lane 2; microsomes treated, in lane 3; cytosol treated, in lane 4. Part D: localization of cucumber LBLOX on microsomes from transgenic tobacco leaves. Following flotation of the membranes in a linear sucrose density gradient, subfractions were analyzed by immunoblot. Lane 1, corresponding to the top of the centrifuge tube (23% sucrose), lane 3 (32% sucrose), lane 5 (39% sucrose), lane 6 (41% sucrose), and lane 7 (sample applied at 43% sucrose).

Fig. 3. *In vitro* experiments showing that radioactively labelled cytosolic LBLOX binds weakly to microsomal membranes (part A) but virtually not to glyoxysomes (part B). Part A: 1 g of cotyledons were incubated with 9 MBq [^{35}S]L-methionine for 3 h. Then, a 100 000 **g** supernatant containing the radioactively labelled cytosolic LBLOX was prepared. This preparation was mixed with a homogenate prepared from untreated cotyledons. The ER/Golgi fraction was isolated by sedimentation and purified by subsequent gradient centrifugation. An aliquot (1/20) of the ER/Golgi fraction (lane 1) and an aliquot (1/20) of the re-isolated cytosol (lane 2) and a large aliquot (1/2) of the ER/Golgi fraction (lane 3) were subjected to SDS-PAGE and fluorography. Part B: in a similar mixing experiment, isolated unlabelled glyoxysomes were incubated with the radioactive cytosol prepared from *in vivo* labelled cotyledons. The subsequent re-isolation of the glyoxysomes and glyoxysomal membranes was performed by flotation in a sucrose gradient. To this means, the incubation mixture was adjusted to 60% (w/w) sucrose. A gradient (56 - 38% sucrose) was layered on top of the sample.

Following centrifugation at 27 000 rpm for 15 h in a Beckman SW-28 rotor, the fractions were analyzed by SDS-PAGE and fluorography. The position in the fluorography of labelled LBLOX is indicated by an arrow. Lanes correspond to the following fraction (their equilibrium densities in parenthesis): Lane 4 (48% sucrose), lane 5 (48,5% sucrose), lane 6 (49% sucrose), lane 7 (50,5% sucrose), lane 8 (52.5% sucrose), lane 24 (56% sucrose), lane 25 (56,5% sucrose), lane 26 (58% sucrose), lane 27 (59% sucrose), and lane 28 (59% sucrose). Fraction numbers 25-28 correspond to the position in the gradient where the suspension was introduced prior to centrifugation. Lanes 4-5 encompass the glyoxysomal membranes, and lane 8 contains, according to the protein profile, the glyoxysomes.

Fig. 4. Affinity of bacterially expressed LBLOX and PLA to liposomes. In 200 µL buffer, 1 µg of the respective proteins were incubated with an amount of liposomes corresponding to 1 mg phosphatidyl choline. After 30 min, the mixture was adjusted to 42% sucrose and placed within a sucrose gradient. Flotation was performed by centrifugation at 100 000 **g** for 6 h. Proteins within subfractions obtained from the gradients were analyzed by SDS-PAGE and immunoblots. For immunodecoration, the respective antisera either raised against LBLOX or against the patatin-like protein were used. The right most lanes always show the bottom position where the incubation mixture was applied to the gradient prior to centrifugation. Thus, flotation was from the right to the left.

Fig. 5. Schematic presentation of the LBLOX structure and its N-terminal part (amino acid residues 48-244) showing the β-barrel. The structure of the LBLOX was calculated on the basis of the crystal structure data obtained for soybean LOX-1 [21] using the primary sequence data of LBLOX. The upper part of the figure is presented in such a way that the N-terminus and the barrel solely consisting of β-sheets is somewhat separated at the lower right side. The main body of LOX encompassing the C-terminal active center is dominated by α-helices. The lower part of the figure provides an

enlarged view of the N[redacted]minal β-barrel. Not shown are the[redacted] amino acid residues of the far N-terminus, an extension not found in other LOX structures. The interrupted structure marked on the lower right side with arrows shows a site where the amino acid sequence of LBLOX substantially deviates from the structure of soybean LOX-1. The program applied (Swiss 3D model, Expasy server) afforded here, similar to LOX-1, a highly flexible loop and thus undefined structure. This loop consists of 14 amino acid residues in LOX-1 but encompasses 20 amino acid residues in the case of LBLOX. The two glutamyl residues E59 and E70 are unique being found only in LBLOX, not in soybean LOX-1. This site might play a role in a Ca^{2+}-coordinated membrane association.

Fig. 6. *In vitro* binding of GST-LBLOX244 fusion protein to isolated lipid bodies. The affinity-purified fusion protein GST-LBLOX244 was added to a suspension of lipid bodies in enriched cytosol. After incubation, lipid bodies were separated from the soluble proteins by flotation. Aliquots of both fractions, and the original crude lipid body suspension (lanes 3 and 6), were analyzed by SDS-PAGE and subsequent immunoblot using anti-LBLOX antiserum. Lanes 2 and 5: lipid bodies recovered by flotation; lanes 1 and 4: soluble proteins re-isolated. The arrow pointing to the band at 51 kDa indicates the fusion protein bound to lipid bodies in excess to the endogenous LBLOX. Lanes 1-3: protein stain; lanes 4-6: immunodecoration.

Fig. 7. Binding to liposomes of LBLOX constructs and recombinant proteins with the wild type and altered β-barrel. After incubation of purified recombinant proteins with liposomes, the liposomes were re-isolated from the incubation mixture by flotation in a sucrose gradient. The behaviour of wild type lipid body LOX (LBLOX) was compared with a fragment containing the N-terminal half of LBLOX (LBLOX-Δ504), the fusion protein with the β-barrel (GST-LOX), and a LBLOX substantially changed in the N-terminal β-barrel (LBLOX-HA_3). The figure shows the analysis of gradient fractions by SDS-PAGE and immunodecoration using anti-LBLOX antiserum.

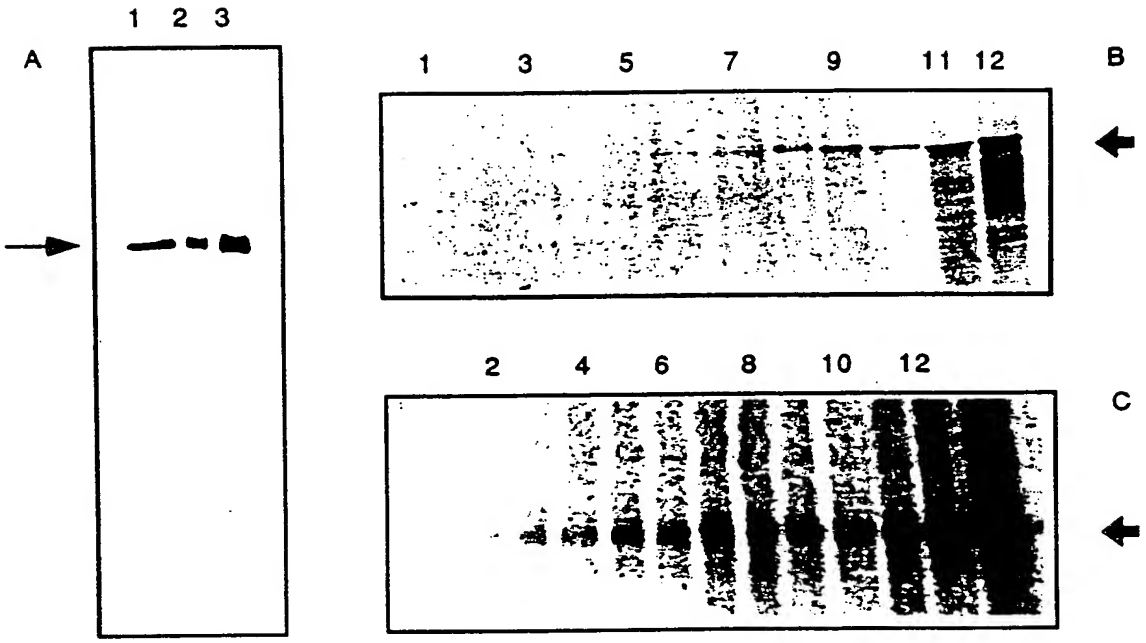
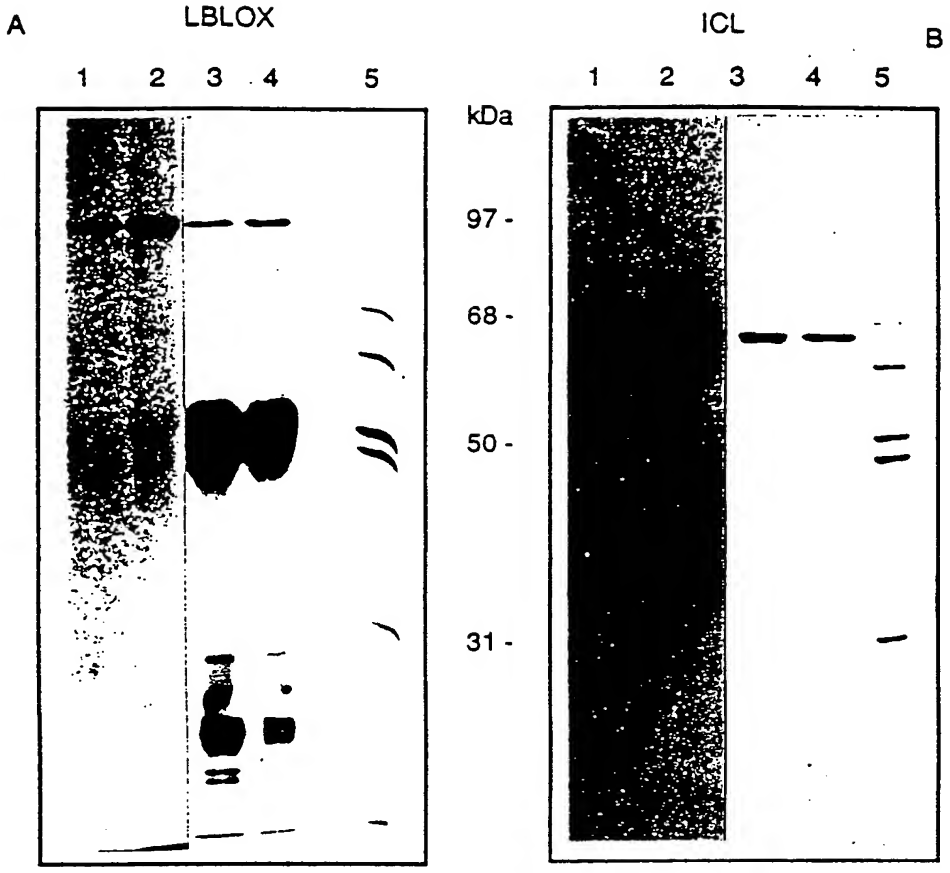
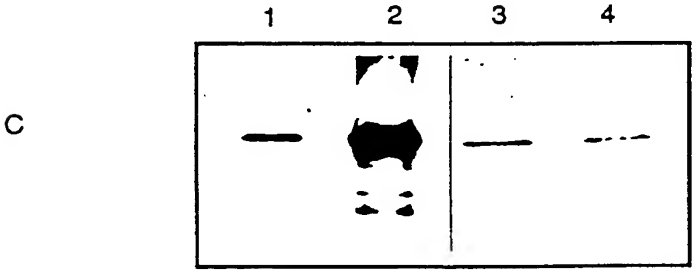


Fig. 1





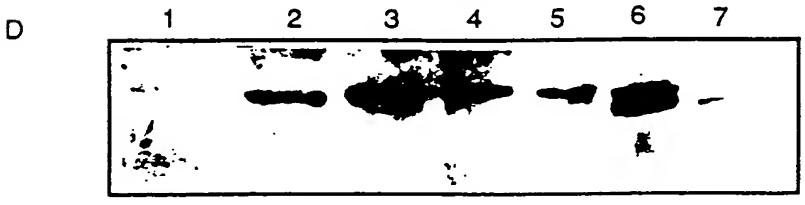
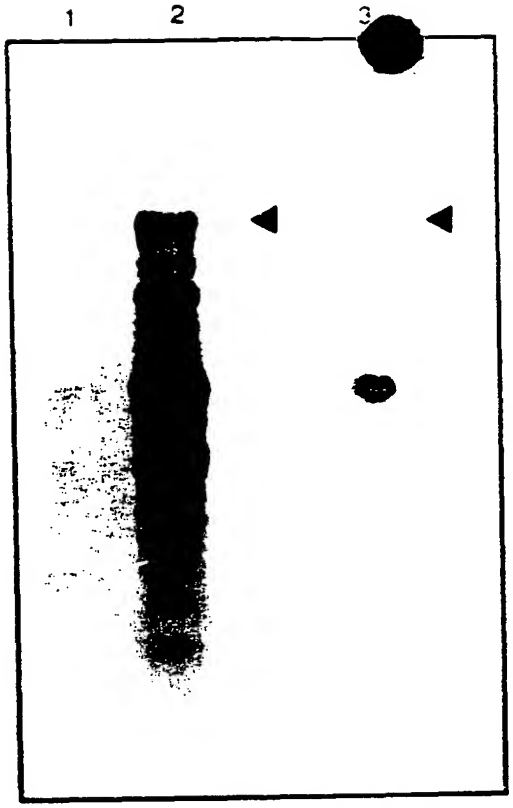


Fig. 2



B

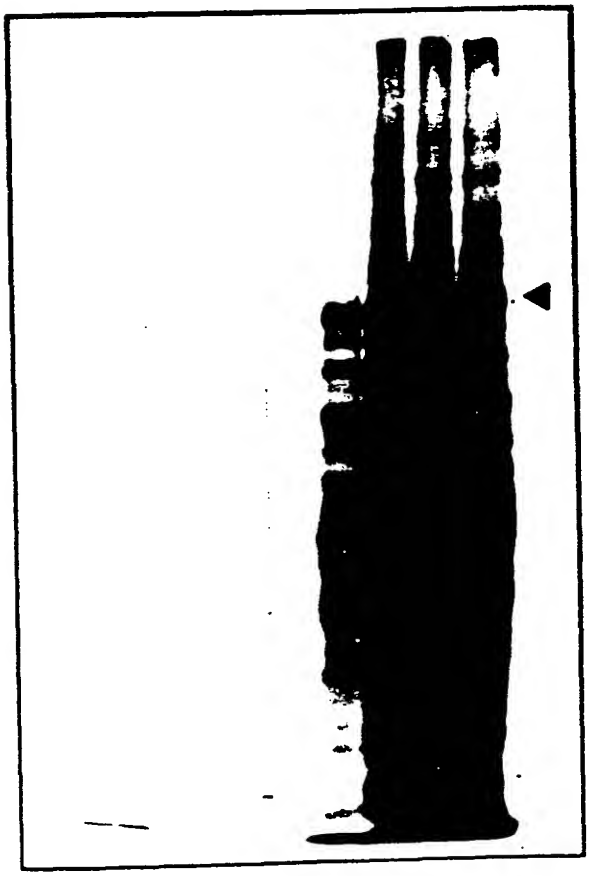


Fig. 3

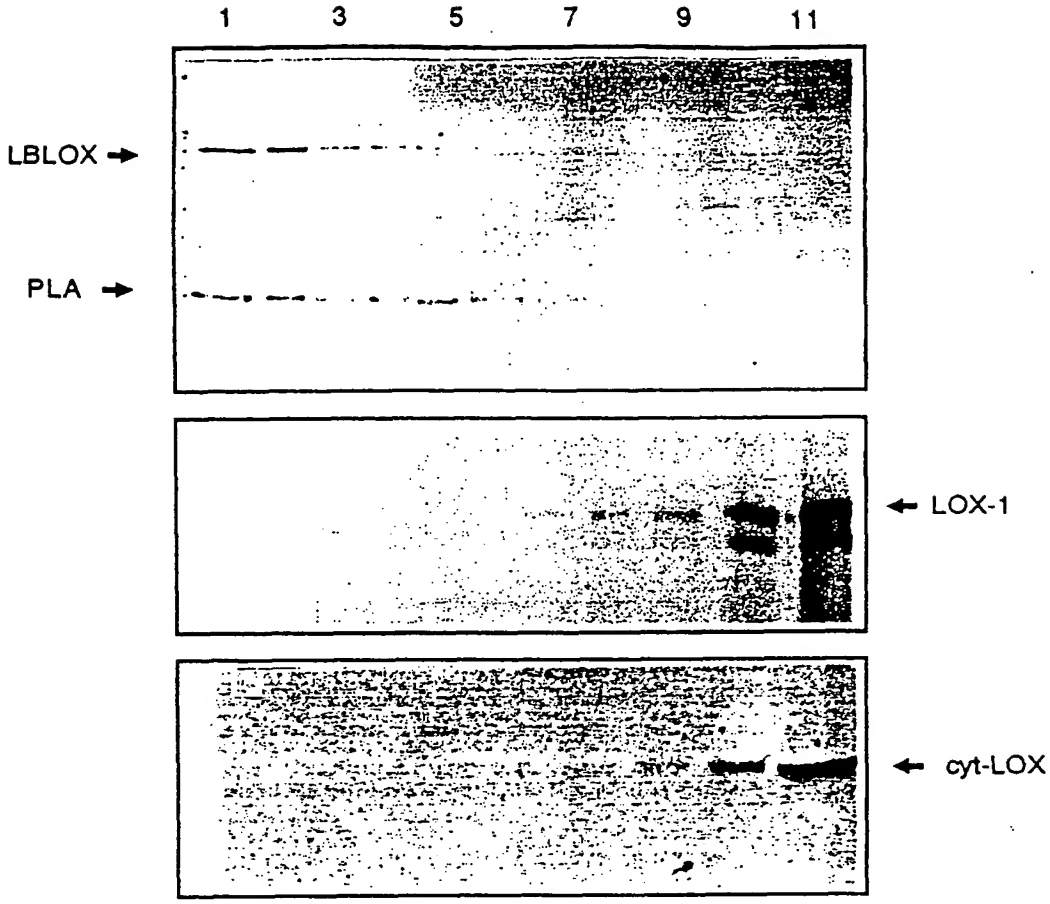
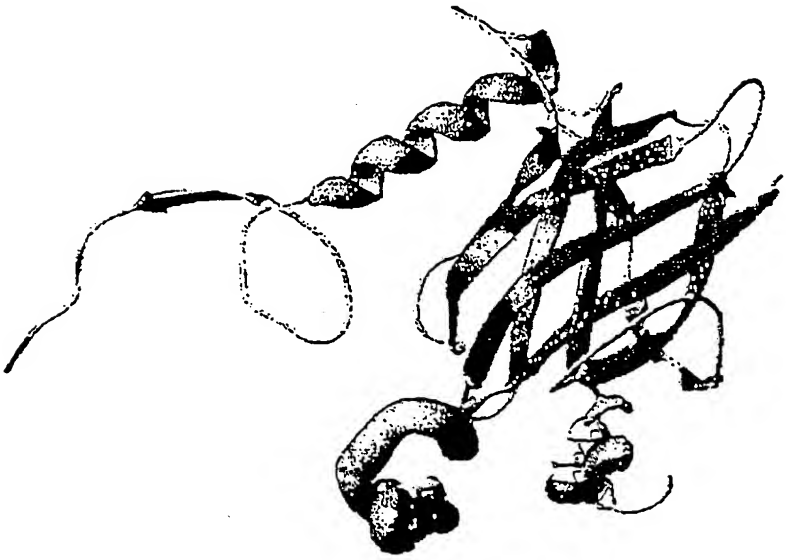


Fig. 4

May et al.



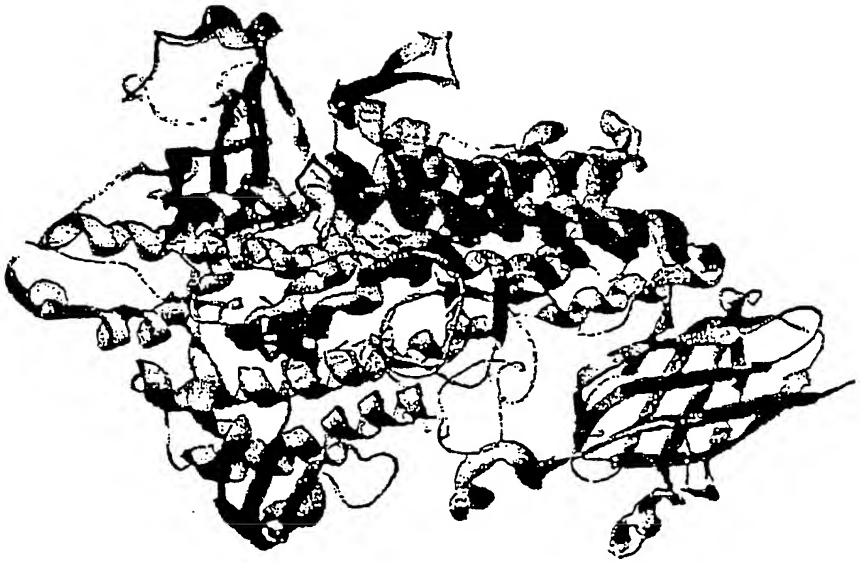


Fig. 5

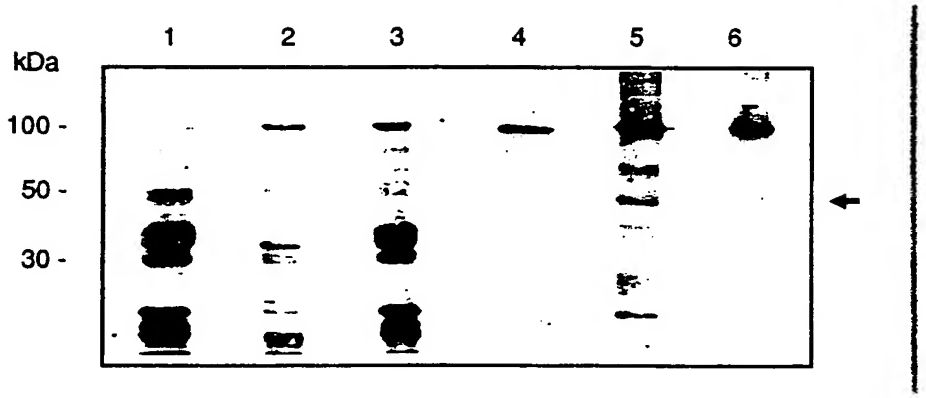
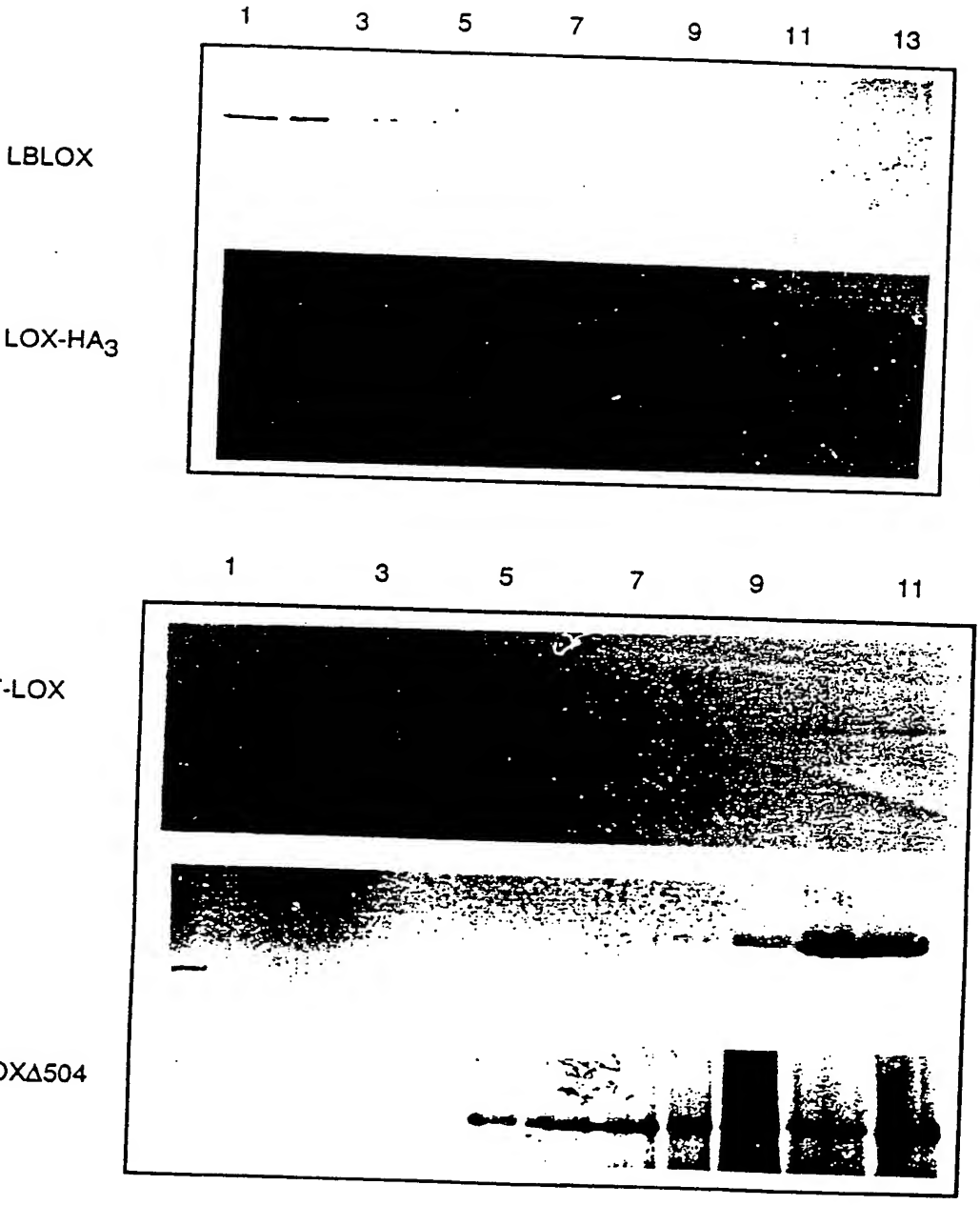


Fig. 6



May et al Fig. 7

SEQUENZPROTOKOLL

<110> BASF Aktiengesellschaft

<120> The N-terminal β-barrel structure of lipid body lipoxygenase mediates its binding to liposomes and lipid bodies

<130> 99_1235

<140>
<141>

<160> 4

<170> PatentIn Vers. 2.0

<210> 1
<211> 732
<212> DNA
<213> Cucumis sativus

<220>
<221> CDS
<222> (1)..(732)

<400> 1

```
atg ttt gga att ggg aag aac atc att gaa ggg gcc ttg aat aca act   48
Met Phe Gly Ile Gly Lys Asn Ile Ile Glu Gly Ala Leu Asn Thr Thr
  1               5                  10                  15

gga gat ctt gca ggt tct gtt atc aat gct ggt ggt aac att tta gat   96
Gly Asp Leu Ala Gly Ser Val Ile Asn Ala Gly Gly Asn Ile Leu Asp
             20                  25                  30

aga gtt tcc agt ctt gga gga aac aaa atc aaa ggg aaa gtg att ctt   144
Arg Val Ser Ser Leu Gly Gly Asn Lys Ile Lys Gly Lys Val Ile Leu
         35                  40                  45

atg aga agc aat gtt ttg gat ttc act gaa ttt cat tcc aat ctt ctt   192
Met Arg Ser Asn Val Leu Asp Phe Thr Glu Phe His Ser Asn Leu Leu
     50                  55                  60

gat aac ttc act gag ctc ttg ggt ggt ggt gtt tct ttc caa ctc att   240
Asp Asn Phe Thr Glu Leu Leu Gly Gly Gly Val Ser Phe Gln Leu Ile
 65                  70                  75                  80

agt gcc act cat act tca aat gac tca aga ggg aaa gtt ggg aac aag   288
Ser Ala Thr His Thr Ser Asn Asp Ser Arg Gly Lys Val Gly Asn Lys
                 85                  90                  95
```

```
gca tat ttg gag agg tgg cta act tca atc cca cca ctg ttt gct gga   336
Ala Tyr Leu Glu Arg Trp Leu Thr Ser Ile Pro Pro Leu Phe Ala Gly
            100                 105                 110

gaa tca gtg ttc caa atc aac ttt caa tgg gat gaa aat ttt gga ttt   384
Glu Ser Val Phe Gln Ile Asn Phe Gln Trp Asp Glu Asn Phe Gly Phe
        115                 120                 125

cca gga gct ttc ttc ata aaa aat gga cat aca agt gaa ttc ttt ctc   432
Pro Gly Ala Phe Phe Ile Lys Asn Gly His Thr Ser Glu Phe Phe Leu
    130                 135                 140

aaa tct ctc act ctt gat gat gtt cct ggc tat ggc aga gtc cat ttt   480
Lys Ser Leu Thr Leu Asp Asp Val Pro Gly Tyr Gly Arg Val His Phe
145                 150                 155                 160

gat tgc aat tct tgg gtt tac cct tct gga aga tac aag aaa gat cgc   528
Asp Cys Asn Ser Trp Val Tyr Pro Ser Gly Arg Tyr Lys Lys Asp Arg
                165                 170                 175

att ttc ttt gcc aat cat gtt tat ctt cca agt caa aca cca aac cct   576
Ile Phe Phe Ala Asn His Val Tyr Leu Pro Ser Gln Thr Pro Asn Pro
            180                 185                 190

ctt cgt aag tat aga gag gaa gaa ttg tgg aat ttg aga gga gat gga   624
Leu Arg Lys Tyr Arg Glu Glu Glu Leu Trp Asn Leu Arg Gly Asp Gly
        195                 200                 205

aca gga gaa aga aag gaa tgg gat aga att tat gac tat gat gtt tat   672
Thr Gly Glu Arg Lys Glu Trp Asp Arg Ile Tyr Asp Tyr Asp Val Tyr
    210                 215                 220

aat gac att gct gac cct gat gtt ggt gat cat cgt cct att ctc ggt   720
Asn Asp Ile Ala Asp Pro Asp Val Gly Asp His Arg Pro Ile Leu Gly
225                 230                 235                 240

ggg acg acc gaa                                                  732
Gly Thr Thr Glu
```

<210> 2
<211> 244
<212> PRT
<213> Cucumis sativus

<400> 2

```
Met Phe Gly Ile Gly Lys Asn Ile Ile Glu Gly Ala Leu Asn Thr Thr
  1               5                  10                  15

Gly Asp Leu Ala Gly Ser Val Ile Asn Ala Gly Gly Asn Ile Leu Asp
            20                  25                  30
```

```
Arg Val Ser Ser Leu Gly Gly Asn Lys Ile Lys Gly Lys Val Ile Leu
         35                  40                  45

Met Arg Ser Asn Val Leu Asp Phe Thr Glu Phe His Ser Asn Leu Leu
     50                  55                  60

Asp Asn Phe Thr Glu Leu Leu Gly Gly Gly Val Ser Phe Gln Leu Ile
 65                  70                  75                  80

Ser Ala Thr His Thr Ser Asn Asp Ser Arg Gly Lys Val Gly Asn Lys
                 85                  90                  95

Ala Tyr Leu Glu Arg Trp Leu Thr Ser Ile Pro Pro Leu Phe Ala Gly
            100                 105                 110

Glu Ser Val Phe Gln Ile Asn Phe Gln Trp Asp Glu Asn Phe Gly Phe
        115                 120                 125

Pro Gly Ala Phe Phe Ile Lys Asn Gly His Thr Ser Glu Phe Phe Leu
    130                 135                 140

Lys Ser Leu Thr Leu Asp Asp Val Pro Gly Tyr Gly Arg Val His Phe
145                 150                 155                 160

Asp Cys Asn Ser Trp Val Tyr Pro Ser Gly Arg Tyr Lys Lys Asp Arg
                165                 170                 175

Ile Phe Phe Ala Asn His Val Tyr Leu Pro Ser Gln Thr Pro Asn Pro
            180                 185                 190

Leu Arg Lys Tyr Arg Glu Glu Glu Leu Trp Asn Leu Arg Gly Asp Gly
        195                 200                 205

Thr Gly Glu Arg Lys Glu Trp Asp Arg Ile Tyr Asp Tyr Asp Val Tyr
    210                 215                 220

Asn Asp Ile Ala Asp Pro Asp Val Gly Asp His Arg Pro Ile Leu Gly
225                 230                 235                 240

Gly Thr Thr Glu
```

<210> 3
<211> 2964
<212> DNA
<213> Cucumis sativus

<220>
<221> CDS

<222> (48)..(2684)

<400> 3

```
gttccaaaca cacagtgagc aaaaaagaaa agtaaaaaag agtgaaa atg ttt gga   56
                                                    Met Phe Gly
                                                      1

att ggg aag aac atc att gaa ggg gcc ttg aat aca act gga gat ctt   104
Ile Gly Lys Asn Ile Ile Glu Gly Ala Leu Asn Thr Thr Gly Asp Leu
      5                  10                  15

gca ggt tct gtt atc aat gct ggt ggt aac att tta gat aga gtt tcc   152
Ala Gly Ser Val Ile Asn Ala Gly Gly Asn Ile Leu Asp Arg Val Ser
 20                  25                  30                  35

agt ctt gga gga aac aaa atc aaa ggg aaa gtg att ctt atg aga agc   200
Ser Leu Gly Gly Asn Lys Ile Lys Gly Lys Val Ile Leu Met Arg Ser
                 40                  45                  50

aat gtt ttg gat ttc act gaa ttt cat tcc aat ctt ctt gat aac ttc   248
Asn Val Leu Asp Phe Thr Glu Phe His Ser Asn Leu Leu Asp Asn Phe
             55                  60                  65

act gag ctc ttg ggt ggt ggt gtt tct ttc caa ctc att agt gcc act   296
Thr Glu Leu Leu Gly Gly Gly Val Ser Phe Gln Leu Ile Ser Ala Thr
         70                  75                  80

cat act tca aat gac tca aga ggg aaa gtt ggg aac aag gca tat ttg   344
His Thr Ser Asn Asp Ser Arg Gly Lys Val Gly Asn Lys Ala Tyr Leu
     85                  90                  95

gag agg tgg cta act tca atc cca cca ctg ttt gct gga gaa tca gtg   392
Glu Arg Trp Leu Thr Ser Ile Pro Pro Leu Phe Ala Gly Glu Ser Val
100                 105                 110                 115

ttc caa atc aac ttt caa tgg gat gaa aat ttt gga ttt cca gga gct   440
Phe Gln Ile Asn Phe Gln Trp Asp Glu Asn Phe Gly Phe Pro Gly Ala
                120                 125                 130

ttc ttc ata aaa aat gga cat aca agt gaa ttc ttt ctc aaa tct ctc   488
Phe Phe Ile Lys Asn Gly His Thr Ser Glu Phe Phe Leu Lys Ser Leu
            135                 140                 145

act ctt gat gat gtt cct ggc tat ggc aga gtc cat ttt gat tgc aat   536
Thr Leu Asp Asp Val Pro Gly Tyr Gly Arg Val His Phe Asp Cys Asn
        150                 155                 160

tct tgg gtt tac cct tct gga aga tac aag aaa gat cgc att ttc ttt   584
Ser Trp Val Tyr Pro Ser Gly Arg Tyr Lys Lys Asp Arg Ile Phe Phe
    165                 170                 175
```

```
gcc aat cat gtt tat ctt cca agt caa aca cca aac cct ctt cgt aag   632
Ala Asn His Val Tyr Leu Pro Ser Gln Thr Pro Asn Pro Leu Arg Lys
180                 185                 190                 195

tat aga gag gaa gaa ttg tgg aat ttg aga gga gat gga aca gga gaa   680
Tyr Arg Glu Glu Glu Leu Trp Asn Leu Arg Gly Asp Gly Thr Gly Glu
                200                 205                 210

aga aag gaa tgg gat aga att tat gac tat gat gtt tat aat gac att   728
Arg Lys Glu Trp Asp Arg Ile Tyr Asp Tyr Asp Val Tyr Asn Asp Ile
            215                 220                 225

gct gac cct gat gtt ggt gat cat cgt cct att ctc ggt ggg acg acc   776
Ala Asp Pro Asp Val Gly Asp His Arg Pro Ile Leu Gly Gly Thr Thr
        230                 235                 240

gaa tat cct tac cct cgt agg gga aga aca gga cga cca cga tca aga   824
Glu Tyr Pro Tyr Pro Arg Arg Gly Arg Thr Gly Arg Pro Arg Ser Arg
    245                 250                 255

aga gac cac aat tat gag agc aga ttg tca cca ata atg agc tta gac   872
Arg Asp His Asn Tyr Glu Ser Arg Leu Ser Pro Ile Met Ser Leu Asp
260                 265                 270                 275

atc tat gta cca aaa gat gaa aac ttt ggg cat ttg aag atg tca gat   920
Ile Tyr Val Pro Lys Asp Glu Asn Phe Gly His Leu Lys Met Ser Asp
                280                 285                 290

ttc ctt ggt tat aca tta aaa gca ctt tcg ata tca atc aaa cca gga   968
Phe Leu Gly Tyr Thr Leu Lys Ala Leu Ser Ile Ser Ile Lys Pro Gly
            295                 300                 305

ctt caa tcc ata ttt gat gta act cca aat gaa ttt gac aat ttt aaa  1016
Leu Gln Ser Ile Phe Asp Val Thr Pro Asn Glu Phe Asp Asn Phe Lys
        310                 315                 320

gaa gtt gat aat ctc ttt gag aga ggt ttt ccc att cca ttt aat gct  1064
Glu Val Asp Asn Leu Phe Glu Arg Gly Phe Pro Ile Pro Phe Asn Ala
    325                 330                 335

ttt aag acc ctc act gag gac ctc act cca cct ttg ttc aaa gca ctc  1112
Phe Lys Thr Leu Thr Glu Asp Leu Thr Pro Pro Leu Phe Lys Ala Leu
340                 345                 350                 355

gtg agg aat gat ggt gaa aaa ttc ctc aaa ttt cct act ccc gaa gtt  1160
Val Arg Asn Asp Gly Glu Lys Phe Leu Lys Phe Pro Thr Pro Glu Val
                360                 365                 370

gtc aaa gat aat aaa ata gga tgg agc act gat gaa gaa ttt gca aga  1208
Val Lys Asp Asn Lys Ile Gly Trp Ser Thr Asp Glu Glu Phe Ala Arg
            375                 380                 385
```

```
gaa atg tta gca gga ccc aat cct cta ttg att cgt cgt ctt gaa gct   1256
Glu Met Leu Ala Gly Pro Asn Pro Leu Leu Ile Arg Arg Leu Glu Ala
        390                 395                 400

ttt cca cca aca agt aag ctt gac cca aat gtt tat ggg aat caa aac   1304
Phe Pro Pro Thr Ser Lys Leu Asp Pro Asn Val Tyr Gly Asn Gln Asn
    405                 410                 415

agt acc atc act gaa gaa cac ata aag cat ggt tta gat ggt ctt acg   1352
Ser Thr Ile Thr Glu Glu His Ile Lys His Gly Leu Asp Gly Leu Thr
420                 425                 430                 435

gtt gat gag gca atg aag caa aac agg ctc tac ata gtg gat ttc cat   1400
Val Asp Glu Ala Met Lys Gln Asn Arg Leu Tyr Ile Val Asp Phe His
                440                 445                 450

gat gca tta atg ccc tat ctt aca agg atg aat gca aca tca aca aaa   1448
Asp Ala Leu Met Pro Tyr Leu Thr Arg Met Asn Ala Thr Ser Thr Lys
            455                 460                 465

aca tat gcc aca aga aca ttg ctt ctt ttg aaa gat gat ggg act ttg   1496
Thr Tyr Ala Thr Arg Thr Leu Leu Leu Leu Lys Asp Asp Gly Thr Leu
        470                 475                 480

aag cca ttg gtt att gag tta gcc ttg cca cat cct caa gga gat caa   1544
Lys Pro Leu Val Ile Glu Leu Ala Leu Pro His Pro Gln Gly Asp Gln
    485                 490                 495

ctt ggt gcc att agc aaa cta tac ttt cca gct gaa aat gga gtt caa   1592
Leu Gly Ala Ile Ser Lys Leu Tyr Phe Pro Ala Glu Asn Gly Val Gln
500                 505                 510                 515

aaa tcc att tgg caa ttg gct aaa gct tat gta act gtt aat gat gtt   1640
Lys Ser Ile Trp Gln Leu Ala Lys Ala Tyr Val Thr Val Asn Asp Val
                520                 525                 530

ggc tac cat caa ctt att agt cat tgg ttg cat act cat gct gta ctt   1688
Gly Tyr His Gln Leu Ile Ser His Trp Leu His Thr His Ala Val Leu
            535                 540                 545

gag cca ttt gtg att gca aca cat aga caa ttg agc gtg ctt cat cca   1736
Glu Pro Phe Val Ile Ala Thr His Arg Gln Leu Ser Val Leu His Pro
        550                 555                 560

atc cat aag ttg ctt gtt cct cat tac aaa gac act atg ttt ata aat   1784
Ile His Lys Leu Leu Val Pro His Tyr Lys Asp Thr Met Phe Ile Asn
    565                 570                 575

gca tct gca aga caa gtt ttg atc aat gcc aat ggt ctt atc gaa aca   1832
Ala Ser Ala Arg Gln Val Leu Ile Asn Ala Asn Gly Leu Ile Glu Thr
```

```
580                 585                 590                 595

acc cat tat cca tca aaa tat tca atg gag ttg tca tct atc ttg tac   1880
Thr His Tyr Pro Ser Lys Tyr Ser Met Glu Leu Ser Ser Ile Leu Tyr
                600                 605                 610

aag gat tgg acc ttc cct gat caa gca tta cct aat aat ctc atg aag   1928
Lys Asp Trp Thr Phe Pro Asp Gln Ala Leu Pro Asn Asn Leu Met Lys
            615                 620                 625

aga gga cta gct gtg gag gac tca agt gcc ccc cat gga ctt aga ttg   1976
Arg Gly Leu Ala Val Glu Asp Ser Ser Ala Pro His Gly Leu Arg Leu
        630                 635                 640

cta ata aat gat tat cca ttt gct gtt gat ggt ctt gac att tgg tca   2024
Leu Ile Asn Asp Tyr Pro Phe Ala Val Asp Gly Leu Asp Ile Trp Ser
    645                 650                 655

gcc att aaa aca tgg gta cag gat tat tgc tgt ctc tac tac aaa gat   2072
Ala Ile Lys Thr Trp Val Gln Asp Tyr Cys Cys Leu Tyr Tyr Lys Asp
660                 665                 670                 675

gac aat gca gta caa aat gac ttt gaa ctc caa tct tgg tgg aat gag   2120
Asp Asn Ala Val Gln Asn Asp Phe Glu Leu Gln Ser Trp Trp Asn Glu
                680                 685                 690

cta aga gag aaa ggc cac gct gac aag aaa cat gaa cca tgg tgg cca   2168
Leu Arg Glu Lys Gly His Ala Asp Lys Lys His Glu Pro Trp Trp Pro
            695                 700                 705

aaa atg caa act tta agt gaa tta atc gaa tcc tgc act aca att ata   2216
Lys Met Gln Thr Leu Ser Glu Leu Ile Glu Ser Cys Thr Thr Ile Ile
        710                 715                 720

tgg att gct tca gct ctt cat gcc gca gtt aac ttt gga caa tat ccc   2264
Trp Ile Ala Ser Ala Leu His Ala Ala Val Asn Phe Gly Gln Tyr Pro
    725                 730                 735

tac gga ggc tat att ctc aat cga cca act aca agt cgt agg ttc atg   2312
Tyr Gly Gly Tyr Ile Leu Asn Arg Pro Thr Thr Ser Arg Arg Phe Met
740                 745                 750                 755

cct gaa gtt ggc acg gct gag tac aaa gaa ctg gaa tcg aat ccc gaa   2360
Pro Glu Val Gly Thr Ala Glu Tyr Lys Glu Leu Glu Ser Asn Pro Glu
                760                 765                 770

aaa gct ttc ttg aga aca ata tgt tca gaa tta caa gca ctt gtt agt   2408
Lys Ala Phe Leu Arg Thr Ile Cys Ser Glu Leu Gln Ala Leu Val Ser
            775                 780                 785

att tca att att gaa atc ttg tca aag cat gct tct gat gaa gtt tat   2456
```

```
Ile Ser Ile Ile Glu Ile Leu Ser Lys His Ala Ser Asp Glu Val Tyr
        790                 795                 800

ctt gga caa aga gct tca att gat tgg act tca gat aaa att gca ttg   2504
Leu Gly Gln Arg Ala Ser Ile Asp Trp Thr Ser Asp Lys Ile Ala Leu
    805                 810                 815

gaa gca ttt gag aaa ttt ggg aaa aat tta ttt gaa gtt gag aat agg   2552
Glu Ala Phe Glu Lys Phe Gly Lys Asn Leu Phe Glu Val Glu Asn Arg
820                 825                 830                 835

atc atg gaa agg aat aaa gag gtg aat ttg aag aat aga tct gga cct   2600
Ile Met Glu Arg Asn Lys Glu Val Asn Leu Lys Asn Arg Ser Gly Pro
                840                 845                 850

gtt aat ttg cct tat act cta ctt gtt cca tca agt aac gaa gga ctc   2648
Val Asn Leu Pro Tyr Thr Leu Leu Val Pro Ser Ser Asn Glu Gly Leu
            855                 860                 865

act gga aga gga att cct aat agt att tct atc taa gttgataaga       2694
Thr Gly Arg Gly Ile Pro Asn Ser Ile Ser Ile
        870                 875

aagaaaagtg gttctttta tgggtgacgt gtgtaatttg aaggtcacaa attacatttt 2754

aagttgccca cattattatt atgaaggaaa taaatgacca tatttttagt ttaatttaaa 2814

ttaggtagct atagccaact ttaggctctg ttggatttgg aactatctcc aacttatata 2874

tgtactttgt actactattt gatgaataaa agttgtgtgt cttaagaata aaaaaaaaaa 2934

aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa                                  2964
```

<210> 4
<211> 878
<212> PRT
<213> Cucumis sativus

<400> 4

```
Met Phe Gly Ile Gly Lys Asn Ile Ile Glu Gly Ala Leu Asn Thr Thr
  1               5                  10                  15

Gly Asp Leu Ala Gly Ser Val Ile Asn Ala Gly Gly Asn Ile Leu Asp
             20                  25                  30

Arg Val Ser Ser Leu Gly Gly Asn Lys Ile Lys Gly Lys Val Ile Leu
         35                  40                  45

Met Arg Ser Asn Val Leu Asp Phe Thr Glu Phe His Ser Asn Leu Leu
     50                  55                  60
```

Asp Asn Phe Thr Glu Leu Leu Gly Gly Gly Val Ser Phe Gln Leu Ile
65 70 75 80

Ser Ala Thr His Thr Ser Asn Asp Ser Arg Gly Lys Val Gly Asn Lys
85 90 95

Ala Tyr Leu Glu Arg Trp Leu Thr Ser Ile Pro Pro Leu Phe Ala Gly
100 105 110

Glu Ser Val Phe Gln Ile Asn Phe Gln Trp Asp Glu Asn Phe Gly Phe
115 120 125

Pro Gly Ala Phe Phe Ile Lys Asn Gly His Thr Ser Glu Phe Phe Leu
130 135 140

Lys Ser Leu Thr Leu Asp Asp Val Pro Gly Tyr Gly Arg Val His Phe
145 150 155 160

Asp Cys Asn Ser Trp Val Tyr Pro Ser Gly Arg Tyr Lys Lys Asp Arg
165 170 175

Ile Phe Phe Ala Asn His Val Tyr Leu Pro Ser Gln Thr Pro Asn Pro
180 185 190

Leu Arg Lys Tyr Arg Glu Glu Glu Leu Trp Asn Leu Arg Gly Asp Gly
195 200 205

Thr Gly Glu Arg Lys Glu Trp Asp Arg Ile Tyr Asp Tyr Asp Val Tyr
210 215 220

Asn Asp Ile Ala Asp Pro Asp Val Gly Asp His Arg Pro Ile Leu Gly
225 230 235 240

Gly Thr Thr Glu Tyr Pro Tyr Pro Arg Arg Gly Arg Thr Gly Arg Pro
245 250 255

Arg Ser Arg Arg Asp His Asn Tyr Glu Ser Arg Leu Ser Pro Ile Met
260 265 270

Ser Leu Asp Ile Tyr Val Pro Lys Asp Glu Asn Phe Gly His Leu Lys
275 280 285

Met Ser Asp Phe Leu Gly Tyr Thr Leu Lys Ala Leu Ser Ile Ser Ile
290 295 300

Lys Pro Gly Leu Gln Ser Ile Phe Asp Val Thr Pro Asn Glu Phe Asp
305 310 315 320

Asn Phe Lys Glu Val Asp Asn Leu Phe Glu Arg Gly Phe Pro Ile Pro
325 330 335

Phe Asn Ala Phe Lys Thr Leu Thr Glu Asp Leu Thr Pro Pro Leu Phe
340 345 350

Lys Ala Leu Val Arg Asn Asp Gly Glu Lys Phe Leu Lys Phe Pro Thr
355 360 365

Pro Glu Val Val Lys Asp Asn Lys Ile Gly Trp Ser Thr Asp Glu Glu
370 375 380

Phe Ala Arg Glu Met Leu Ala Gly Pro Asn Pro Leu Leu Ile Arg Arg
385 390 395 400

Leu Glu Ala Phe Pro Pro Thr Ser Lys Leu Asp Pro Asn Val Tyr Gly
405 410 415

Asn Gln Asn Ser Thr Ile Thr Glu Glu His Ile Lys His Gly Leu Asp
420 425 430

Gly Leu Thr Val Asp Glu Ala Met Lys Gln Asn Arg Leu Tyr Ile Val
435 440 445

Asp Phe His Asp Ala Leu Met Pro Tyr Leu Thr Arg Met Asn Ala Thr
450 455 460

Ser Thr Lys Thr Tyr Ala Thr Arg Thr Leu Leu Leu Leu Lys Asp Asp
465 470 475 480

Gly Thr Leu Lys Pro Leu Val Ile Glu Leu Ala Leu Pro His Pro Gln
485 490 495

Gly Asp Gln Leu Gly Ala Ile Ser Lys Leu Tyr Phe Pro Ala Glu Asn
500 505 510

Gly Val Gln Lys Ser Ile Trp Gln Leu Ala Lys Ala Tyr Val Thr Val
515 520 525

Asn Asp Val Gly Tyr His Gln Leu Ile Ser His Trp Leu His Thr His
530 535 540

Ala Val Leu Glu Pro Phe Val Ile Ala Thr His Arg Gln Leu Ser Val
545 550 555 560

Leu His Pro Ile His Lys Leu Leu Val Pro His Tyr Lys Asp Thr Met
565 570 575

Phe Ile Asn Ala Ser Ala Arg Gln Val Leu Ile Asn Ala Asn Gly Leu
580 585 590

Ile Glu Thr Thr His Tyr Pro Ser Lys Tyr Ser Met Glu Leu Ser Ser
595 600 605

Ile Leu Tyr Lys Asp Trp Thr Phe Pro Asp Gln Ala Leu Pro Asn Asn
610 615 620

Leu Met Lys Arg Gly Leu Ala Val Glu Asp Ser Ser Ala Pro His Gly
625 630 635 640

Leu Arg Leu Leu Ile Asn Asp Tyr Pro Phe Ala Val Asp Gly Leu Asp
645 650 655

Ile Trp Ser Ala Ile Lys Thr Trp Val Gln Asp Tyr Cys Cys Leu Tyr
660 665 670

Tyr Lys Asp Asp Asn Ala Val Gln Asn Asp Phe Glu Leu Gln Ser Trp
675 680 685

Trp Asn Glu Leu Arg Glu Lys Gly His Ala Asp Lys Lys His Glu Pro
690 695 700

Trp Trp Pro Lys Met Gln Thr Leu Ser Glu Leu Ile Glu Ser Cys Thr
705 710 715 720

Thr Ile Ile Trp Ile Ala Ser Ala Leu His Ala Ala Val Asn Phe Gly
725 730 735

Gln Tyr Pro Tyr Gly Gly Tyr Ile Leu Asn Arg Pro Thr Thr Ser Arg
740 745 750

Arg Phe Met Pro Glu Val Gly Thr Ala Glu Tyr Lys Glu Leu Glu Ser
755 760 765

Asn Pro Glu Lys Ala Phe Leu Arg Thr Ile Cys Ser Glu Leu Gln Ala
770 775 780

Leu Val Ser Ile Ser Ile Ile Glu Ile Leu Ser Lys His Ala Ser Asp
785 790 795 800

Glu Val Tyr Leu Gly Gln Arg Ala Ser Ile Asp Trp Thr Ser Asp Lys
805 810 815

Ile Ala Leu Glu Ala Phe Glu Lys Phe Gly Lys Asn Leu Phe Glu Val
820 825 830

Glu Asn Arg Ile Met Glu Arg Asn Lys Glu Val Asn Leu Lys Asn Arg
835 840 845

Ser Gly Pro Val Asn Leu Pro Tyr Thr Leu Leu Val Pro Ser Ser Asn
850 855 860

Glu Gly Leu Thr Gly Arg Gly Ile Pro Asn Ser Ile Ser Ile
865 870 875